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Original Article

Gentamicin-induced readthrough and nonsense-mediated mRNA decay of *SERPINB7* nonsense mutant transcripts

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Abbreviations: EJC, exon-junction complex; FLuc, firefly luciferase; NHEK, normal human epidermal keratinocyte; NMD, nonsense-mediated mRNA decay; NPPK,

Nagashima-type palmoplantar keratosis; PABPC1, poly(A)-binding protein cytoplasmic 1; PTC, premature termination codon; siRNA, small interfering RNA; SEM, standard error of the mean; UPF1, up-frameshift protein 1; VAS, visual analog scale

ABSTRACT

Nagashima-type palmoplantar keratosis (NPPK) is an autosomal recessive skin disorder with a high, unmet medical need that is caused by mutations in *SERPINB7*. Almost all NPPK patients carry the founder nonsense mutation c.796C>T (p.Arg266Ter) in the last exon of *SERPINB7*. Here we sought to determine whether topical "nonsense-suppression (readthrough)" therapy using gentamicin is applicable to NPPK. First, we demonstrated that gentamicin enhanced readthrough activity in cells transfected with *SERPINB7* cDNA carrying the mutation and promoted full-length SERPINB7 protein synthesis in NPPK keratinocytes. We next conducted an investigator-blinded, randomized, bilaterally controlled compassionate use study of topical gentamicin in which five NPPK patients with c.796C>T were enrolled. Patients' selfreported improvement of hyperkeratosis was significantly greater on the gentamicin side than the control side (P=0.0349). In two patients, hyperkeratosis was improved on the gentamicin side, as determined by a blinded-investigator assessment. These results indicate the therapeutic potential of topical gentamicin for NPPK. Unexpectedly, we also found that mutant *SERPINB7* mRNAs harboring r.796c>u were degraded by

nonsense-mediated mRNA decay. Furthermore, the truncated SERPINB7 protein was degraded via a proteasome-mediated pathway. These findings provide important insights into the mRNA/protein quality control system in humans, which could be a potential therapeutic target for genetic diseases. (199 words)

INTRODUCTION

Nagashima-type palmoplantar keratosis (NPPK; MIM#615598) is the most common form of palmoplantar keratodermas in East Asian populations and is inherited in an autosomal recessive fashion (Kubo et al., 2013; Kubo et al., 2014). NPPK is clinically characterized by diffuse and “transgressive” hyperkeratosis and erythema on the palms and soles extending to the dorsum of the hands and the feet, the flexor aspects of the wrists and the ankles, and the Achilles tendon areas, often involving the elbows and knees (Kabashima et al., 2009; Kubo et al., 2013). Loss-of-function mutations (mostly nonsense mutations) in the gene encoding SERPINB7, a member of the serine protease inhibitor superfamily highly expressed in the stratum granulosum, have been shown to cause NPPK (Kubo et al., 2013). However, there is still no curative therapy for NPPK, and current disease management strategies primarily aim to reduce hyperkeratosis using topical vitamin D3 and/or topical keratolytics, such as salicylic acid, urea, and adapalene, which produce unsatisfactory outcomes.

Nonsense mutations account for approximately 11% and 20% of disease-causing gene

lesions and single-base-pair mutations, respectively, with some genes showing considerably higher nonsense mutation frequencies (Mort et al., 2008). Nonsense mutations create a premature termination codon (PTC)—UGA, UAG, or UAA—which usually triggers degradation of PTC-containing mRNAs by mRNA quality control pathways to reduce the production of a potentially deleterious truncated protein. PTC recognition is largely mediated by protein complexes called exon-junction complexes (EJCs), which are deposited 20-24 nucleotides upstream of exon-exon junctions during pre-mRNA splicing (Le Hir et al., 2000; Shoemaker and Green., 2012). In the pioneer round of translation, ribosomes displace EJCs, and a PTC is distinguished from a canonical stop codon by the presence of one or more EJCs downstream of a stop codon (Maquat et al., 2010; Shoemaker and Green., 2012). PTC-containing mRNAs are then subjected to nonsense-mediated mRNA decay (NMD). Importantly, the first step of this process is mediated by the recruitment of up-frameshift protein 1 (UPF1), which is a key NMD factor, by the ribosome stalled on a PTC (Kurosaki and Maquat., 2016). Because NMD reduces the PTC-containing mRNA level to ~5–25% of the PTC-free mRNA level, ribosomes translate only mutant transcripts that escape NMD into

truncated protein (Kervestin and Jacobson., 2012; Kuzmiak and Maquat., 2006). Importantly, translational termination by a PTC is not 100% efficient, and decoding of the stop codon by a near-cognate tRNA occurs at a low frequency (Bidou et al., 2012). This natural suppression of PTCs, so-called “readthrough”, can lead to the restoration of a full-length protein (Bidou et al., 2012). Indeed, the therapeutic potential of readthrough-enhancing drugs, including aminoglycoside antibiotics (gentamicin, G418, paromomycin, and amikacin) and ataluren (Translarna®, formerly known as PTC124), has been verified in cell-based assays, cultured mammalian cells, animal models, and even human patients with nonsense mutations (Barton-Davis et al., 1999; Bidou et al., 2012; Roy et al., 2016; Welch et al., 2007; Wilschanski et al., 2003). Nevertheless, the clinical usage of these drugs for genetic diseases remains very limited, largely because systemic aminoglycosides have renal and otic toxicities, and these readthrough agents show considerably varying readthrough efficiencies among the target nonsense mutations (Manuvakhova et al., 2000). Notably, the levels of readthrough for a given nonsense mutation mainly depend on the context of the stop codon (UGA > UAG > UAA) and the identity of the nucleotide immediately downstream from the stop codon

(for example, UGAC > UGAA > UGAU, UGAG) (Manuvakhova et al., 2000). Furthermore, higher mutant transcript levels ensure more synthesis of readthrough-generated full-length protein, i.e., the degradation of PTC-containing mRNAs by NMD represents a limitation of nonsense suppression strategies (Bidou et al., 2012; Linde et al., 2007).

We hypothesized that NPPK represents an ideal readthrough target because more than 90% of the reported patients carry the TGAA nonsense mutation c.796C>T (Hashimoto et al., 2017; Hida et al., 2015; Kubo et al., 2013; Li et al., 2016; Miyauchi et al., 2016; Mizuno et al., 2014; Nakajima et al., 2016; On et al., in press; Shiohama et al., 2015; Suzuki et al., 2015; Yin et al., 2014; Zhang et al., 2016), and the mutant mRNA is likely to escape NMD, as it occurs in the last exon of the gene. The estimated number of NPPK patients carrying c.796C>T is extremely large—at least 0.15 million in Japan and China—because the allele frequency of this mutation is higher than 0.01 in the Japanese and Chinese populations (Kubo et al., 2013), which again makes the mutation a very attractive therapeutic target. Furthermore, given that SERPINB7 is highly expressed by

keratinocytes in the stratum granulosum (Kubo et al., 2013), topical application of readthrough-promoting compounds appears to elicit full-length protein synthesis. Because production of a small amount of functional protein has been shown to be sufficient for clinical improvement in a number of autosomal recessive disorders such as McArdle disease (Haller and Vissing., 2002), even partial restoration of full-length SERPINB7 by readthrough agents should attenuate the NPPK phenotype.

In this study, we investigated the potential of gentamicin and ataluren to promote readthrough of c.796C>T in *SERPINB7 in vitro* and demonstrated that gentamicin suppresses the nonsense mutation in a dose-dependent manner. We also performed a "proof-of-concept" compassionate use study of topical gentamicin for NPPK, which showed that a four-week application of gentamicin ameliorated its clinical phenotypes. These results collectively highlight the therapeutic potential of topical gentamicin for NPPK. Furthermore, we unexpectedly found that mutant *SERPINB7* mRNAs harboring the mutation were degraded by NMD and that truncated SERPINB7 was digested by a proteasome-mediated pathway. Because little is known about the mechanisms

contributing to the degradation of mRNAs with a nonsense mutation in the last exon of a human gene and its protein products, these findings should expand our understanding of the mRNA/protein quality control system and facilitate a novel therapeutic approach for the treatment of genetic diseases.

RESULTS

Gentamicin restores full-length SERPINB7 via c.796C>T readthrough in *SERPINB7* cDNA

We first sought to determine whether gentamicin can promote readthrough of the nonsense mutation c.796C>T in *SERPINB7 in vitro*. Mutant *SERPINB7* cDNA harboring c.796C>T was transfected into AD293 cells together with a firefly luciferase (FLuc) gene, which was used to normalize transfection efficiency (Figure 1a). In theory, the baseline production of full-length SERPINB7 is close to zero, whereas its production would increase if gentamicin allows ribosomes to ignore the PTC (Figure 1b). The cells were subsequently treated with eight different concentrations of gentamicin (39-10,000 μg/ml). Gentamicin restored the production of full-length

SERPINB7 at concentrations between 312 and 5,000 μg/ml in a dose-dependent manner, while the protein signal at 10,000 μg/ml slightly decreased compared with that at 5,000 μg/ml (Figure 1c). By contrast, gentamicin did not increase FLuc production. Thus, we clearly demonstrated that gentamicin can promote readthrough of c.796C>T in *SERPINB7* cDNA.

Ataluren does not promote readthrough of c.796C>T in *SERPINB7* cDNA

AD293 cells transiently transfected with the mutant *SERPINB7* cDNA were similarly treated with a broad range of concentrations of ataluren (1.56-100 μM). Intriguingly, ataluren was not able to restore full-length SERPINB7 production at any concentration tested (Figure 1c). Rather, ataluren enhanced the FLuc signal, probably by increasing protein stability (Figure 1c).

Gentamicin enhances full-length SERPINB7 protein production in NPPK keratinocytes

Next, we investigated the readthrough efficiency of gentamicin using immortalized

primary keratinocytes derived from an NPPK patient homozygous for c.796C>T (P1 in the Table 1). The immortalized NPPK keratinocytes were treated with 10,000 or 15,000 μg/ml of gentamicin. The cell lysates were subjected to Western blotting analysis using an antibody against the N-terminus of SERPINB7 to detect full-length SERPINB7. Because a number of non-specific bands were observed when this antibody was used to analyze keratinocyte lysates, we first characterized the antibody to differentiate between specific and non-specific bands using small interfering RNA (siRNA) knockdown of *SERPINB7* and identified a SERPINB7-specific band (Figure 2). Using this antibody, we found that gentamicin treatment restored full-length SERPINB7 at both concentrations (Figure 2), although it reduced the cell viability of keratinocytes at these concentrations (data not shown). In contrast, little SERPINB7 was detected when the cells were untreated (Figure 2). Thus, we confirmed that gentamicin suppresses c.796C>T in NPPK patient-derived keratinocytes.

Compassionate use study of topical gentamicin in NPPK patients with c.796C>T

To verify the *in vivo* readthrough potential of gentamicin, we next conducted an

investigator-blinded, randomized, bilaterally controlled compassionate use study of topical gentamicin. In brief, five NPPK patients (Table 1)—three homozygotes for c.796C>T and two compound heterozygotes for c.796C>T and c.455-1G>A—were instructed to apply 0.1% gentamicin ointment, which is made from Vaseline, on one hand and Vaseline alone as a control on the other hand for four weeks, and the side that received each treatment was randomly assigned. Because one homozygote (P5 in the Table 1) reported to us on day 21 that pruritic erythema and vesicles had appeared exclusively on the dorsum of the gentamicin-applied hand on day 14, he was asked to stop using gentamicin ointment on day 21 and thereafter. Considering that he was patch-test negative to gentamicin and that pruritic erythema and vesicles were not observed on the gentamicin-applied palm, we concluded that he had developed acute eczema, but not contact dermatitis. Topical gentamicin was well tolerated by the remaining four patients, as they showed no clinical signs of systemic or local toxicity. Notably, the gentamicin side showed a significantly greater reduction in visual analog scale (VAS) scores with regard to hyperkeratosis than the control side after a four-week treatment (mean ± standard error of the mean (SEM); 19 ± 3.32 vs 3 ± 2; P=0.0349;

Figure 3a and Supplementary Table 1). By a blinded-investigator assessment, hyperkeratosis was improved on the gentamicin side in two homozygotes (P1 and P5 in the Table 1) (Figure 3b and Supplementary Figure S1). Reduced scaling and smoothening of their palmar skin surfaces were also noted (Figure 3b and Supplementary Figure S1). Although topical gentamicin did not improve the degree of erythema by either subjective or objective assessment in any of the patients (Supplementary Table S2), subjective and objective assessments illustrated that it suppressed hyperkeratosis in four and two patients by, respectively.

Degradation of mutant *SERPINB7* mRNA by NMD

Because c.796C>T is located in the last exon of *SERPINB7*, we initially hypothesized that the mutant mRNA might not be degraded by NMD in keratinocytes carrying the mutation. To validate this hypothesis, we first determined the expression levels of *SERPINB7* mRNA in the skin of eight individuals—five were homozygous and three were wild type for c.796C>T—using quantitative real-time reverse transcriptase PCR. Unexpectedly, the expression levels of *SERPINB7* mRNA in the homozygotes were

47% lower than those in the wild-type individuals, and this difference was statistically significant (P=0.00069) (Figure 4a). To address the mechanism underlying the decrease, we next treated immortalized NPPK keratinocytes with cycloheximide (CHX), an inhibitor of translation, and quantified the mutant transcript levels. Notably, CHX-treated NPPK keratinocytes showed a greater increase in nonsense-containing *SERPINB7* mRNA levels than CHX-treated immortalized normal human epidermal keratinocytes (NHEKs; Figure 4b), which clearly indicates that translation-coupled mRNA degradation is involved in this process. Furthermore, siRNA knockdown of *UPF1* led to a statistically significant increase in the stability of mutant *SERPINB7* mRNA in immortalized NPPK keratinocytes compared with cells transfected with a scramble siRNA (P=0.00157 and 0.00022 for siRNA#1 and siRNA#2, respectively) (Figure 4c). In contrast, *UPF1* knockdown did not increase *SERPINB7* mRNA levels in the immortalized NHEKs (Figure 4c). These data collectively indicate that NMD occurs for the nonsense mutation c.796C>T, despite the mutant transcript lacking a downstream EJC (EJC-independent NMD).

Proteasome-mediated degradation of truncated SERPINB7 protein

When we transfected the mutant and wild-type *SERPINB7* cDNA into HaCaT cells, we found that the protein level of truncated SERPINB7 was greatly reduced compared with that of full-length SERPINB7 (Figures 1a and 5a). Although EJC-independent NMD alone may explain the lower protein expression in the mutant-transfected cells, we wondered whether additional proteasome-mediated protein degradation might underlie this process. To address this, we measured the protein levels of SERPINB7 after treating the mutant-transfected cells with MG132, a 26S proteasome inhibitor. Notably, the addition of MG132 increased protein levels, whereas MG132 treatment did not alter protein levels in the wild-type-transfected cells (Figure 5a). Similarly, treatment of immortalized NPPK keratinocytes with MG132 dose-dependently increased the production of truncated SERPINB7 (Figure 5b). These results collectively led us to conclude that the truncated SERPINB7 proteins synthesized from PTC-containing transcripts are regulated by the 26S proteasome-mediated protein degradation pathway.

DISCUSSION

In this study, we clearly demonstrated that gentamicin suppresses c.796C>T in cells transiently transfected with the mutant *SERPINB7* cDNA and in NPPK keratinocytes. These results suggest that c.796C>T is a “good responder” mutation to readthrough therapy using gentamicin. Furthermore, topical 0.1% gentamicin ameliorated hyperkeratosis in patients with NPPK, indicating its promise for the treatment of NPPK. Before this study, no randomized controlled clinical study had been performed using topical readthrough agents. There was only one report of a non-blinded, non-randomized, single-patient clinical study of topical gentamicin for Hailey-Hailey disease (MIM#169600), an autosomal dominant blistering disease, where gentamicin yielded slightly better clinical improvements of a submammary skin eruption in a patient harboring a heterozygous TGA nonsense mutation (c.1402C>T; p.Arg468Ter) in *ATP2C1* compared with a topical preparation containing 5% boric acid and 2% salicylic acid (Kellermayer et al., 2006). In revising our manuscript, we learned that a double-blind, placebo-controlled pilot trial of gentamicin for recessive dystrophic epidermolysis bullosa (MIM#226600) was recently published (Woodley et al., 2017). In this study, five patients carrying nonsense mutations in *COL7A1* showed a significant

increase in type VII collagen expression via topical or intradermal administration of the drug. Thus, we provide further “proof-of-concept” clinical data supporting the therapeutic potential of topical gentamicin in an autosomal recessive disorder. However, an important question—what percentage of physiological SERPINB7 protein level provides reasonable epidermal homeostasis—has yet to be answered. In our compassionate use study, improvement of erythema was not observed in any participant, which suggests the following two possibilities: (1) More synthesis of SERPINB7 is required to completely cure the disease, or (2) some of gentamicin-induced SERPINB7 may not be fully functional because aminoglycosides induce ribosomes to readthrough PTCs via the incorporation of random amino acids by near-cognate aminoacyl tRNAs. Nevertheless, the regression of hyperkeratosis seen on the gentamicin-treated hands is sufficiently promising to warrant a larger confirmatory trial of topical gentamicin in NPPK and other genetic skin disorders caused by nonsense mutations with limited therapeutic options. Furthermore, a longer treatment and observation period should be considered in future studies. Given that gentamicin is associated with nephrotoxicity and ototoxicity, one could hypothesize that a long use of topical gentamicin would

result in these severe adverse events. However, this does not appear to be the case with NPPK, because the topical daily dose of 0.1% gentamicin ointment used in our study was much lower than that used in previously reported clinical studies, in which systemic gentamicin exhibited neither nephrotoxicity nor ototoxicity (Malik et al., 2010); this would be the case even if 100% of the topical gentamicin had entered the patients' bloodstream. Rather, we speculate that allergic skin reactions may represent a limitation of the therapeutic application of topical gentamicin, considering its allergic potential (Gehrig and Warshaw., 2008).

In contrast, this study demonstrated that ataluren did not enhance the production of full-length SERPINB7 from the mutant cDNA carrying c.796C>T at concentrations of 1.56-100 μM, indicating that ataluren is not capable of suppressing the mutation. Ataluren was originally identified by high-throughput screening for small molecules that increased the frequency of readthrough of a UGA PTC in an FLuc gene, possibly by promoting the insertion of a near-cognate tRNA at the PTC site (Roy et al., 2016; Welch et al., 2007). Although more than 20 nonsense suppression experiments in

cell-based or animal models have suggested the readthrough ability of ataluren (Peltz et al., 2013; Roy et al., 2016), our results challenge this view. In support of our results, studies have cast doubt on its efficacy and its underlying mechanism of action (Auld et al., 2009; Auld et al., 2010; Brumm et al., 2012; McElroy et al., 2013). Ataluren could not rescue the two PTCs (UGAG and UAGU) in the collagen type VII gene (*COL7A1*) at concentrations of 0.01-100 μM (McElroy et al., 2013) or the four PTCs (UGAA, UAAU, UAGA, and UAAG) in the melanocortin 4 receptor gene (*MCR4*) at concentrations of 7.5 or 75 μg/ml (Brumm et al., 2012), whereas aminoglycosides did enhance readthrough of these PTCs. A phase 3 trial in cystic fibrosis also failed to hit its primary endpoint and reach statistical significance (Kerem et al., 2014). Furthermore, ataluren's ability to suppress nonsense codons may be an artifact of the FLuc reporter assay that was used to discover the drug, because it stabilizes FLuc, an enzyme that is otherwise rapidly degraded (Auld et al., 2009; Auld et al., 2010; McElroy et al., 2013). Although identifying the precise molecular target of ataluren could resolve these issues, this study demonstrated that ataluren does not suppress c.796C>T in *SERPINB7* but increases FLuc protein levels.

NMD triggered by c.796C>T in the last exon of *SERPINB7* is intriguing because the EJC-dependent PTC recognition model predicts that mRNAs with a nonsense mutation in a terminal exon of a given gene are stable, as they lack a downstream EJC, which defines the stop codon as premature (Kurosaki and Maquat., 2016). In human diseases, EJC-independent NMD has been experimentally verified only in patients with Schmid metaphyseal chondrodysplasia (MIM#156500) carrying three specific PTC mutations—p.Tyr632Ter, p.Trp651Ter and p.Tyr663Ter—in the last exon of *COL10A1*, a gene encoding type X collagen (Fang et al., 2013). Given that PTC recognition by an aberrantly long 3′ untranslated region (UTR) can activate NMD in yeast, *Drosophila melanogaster*, *Caenorhabditis elegans*, and plant and mammalian cells (Amrani et al., 2004; Bühler et al., 2006; Kuroha et al., 2009; Kurosaki and Maquat., 2016), one plausible explanation for the unexpected *SERPINB7* NMD is a 342-bp extension of its native 741-bp 3′ UTR resulting from c.796C>T (Figure 1a). An extended 3′ UTR that is devoid of EJC increases physical distance between the terminating ribosome and the mRNA 3′ poly(A) tail and reduces the interaction between eukaryotic release factor 3 at

the PTC and cytoplasmic poly(A)-binding protein 1 (PABPC1), resulting in NMD (Fang et al., 2013). The importance of the proximity between PABPC1 and a termination codon in NMD has been further supported by showing that tethering PABPC1 to a position sufficiently close to the 3D environment of a termination codon can inhibit NMD (Amrani et al., 2004). Thus, impaired translation termination at a PTC by an extended 3′ UTR can trigger the rapid degradation of PTC-containing mRNA (the so-called “faux 3′ UTR” model) (Amrani et al., 2004; Kurosaki and Maquat., 2016). Thus, we speculate that the extended 1083-bp 3′ UTR of the mutant *SERPINB7* might make the mutant transcript a substrate for NMD. The involvement of the 3′ UTR in recognizing PTCs in genes that have a large 3′ terminal exon might represent a “fail-safe” RNA decay pathway to eliminate mutant transcripts that would otherwise escape degradation and result in C-terminally truncated proteins with potentially dominant-negative deleterious effects. Interestingly, EJC-independent NMD has been suggested to be less efficient than EJC-dependent NMD (Kurosaki and Maquat., 2016). This may explain why the expression level of SERPINB7 mRNA in the c.796C>T homozygotes was only 47% lower than that in the wild-type individuals in this study,

whereas EJC-dependent NMD generally reduces the PTC-containing mRNA levels to ~5–25% of the wild-type levels (Kervestin and Jacobson., 2012; Kuzmiak and Maquat., 2006).

Notably, we also demonstrated that truncated SERPINB7 protein from the mutant r.796c>u transcripts is degraded by the 26S proteasome. In yeast, mRNAs harboring a faux 3′ UTR stimulate Upf1-dependent proteasome-mediated degradation of the PTC products (Kuroha et al., 2009; Kuroha et al., 2013; Sugiyama et al., 2017). These findings collectively indicate that mRNA and protein stability might be regulated by the aberrantly long 3′ UTR of mutant transcripts in humans. The nature of *SERPINB7* mRNA decay should be further clarified in a future study, which may provide important insights into the mRNA/protein surveillance pathways in humans. Furthermore, unveiling NMD mechanisms that are specific to keratinocytes is worthwhile, because cell type specificity with regard to NMD has been reported (Bateman et al., 2003; Carter et al., 1996). Inhibition of keratinocyte-specific NMD would benefit patients with a broad variety of genodermatoses, including NPPK, by tissue-specifically

enhancing PTC readthrough.

MATERIALS AND METHODS

For extended Materials and Methods, please refer to the Supplementary Materials online. All *in vitro* assays and quantitative mRNA analyses were performed three or more times.

Clinical study

Eligible participants had histologically and genetically confirmed NPPK. Five participants were randomly assigned to application of 0.1% gentamicin ointment (MSD, Tokyo, Japan) on one hand and Vaseline (Pfizer, Tokyo, Japan) on the other hand (split-body design) and were requested to apply 0.5 g of each topical agent twice daily for 4 weeks. Primary endpoints were patient and investigator assessments of the intensity of erythema and the degree of hyperkeratosis for the gentamicin-applied hand and Vaseline-applied hand. For patient assessments, ratings were recorded on a 0-100 mm VAS before and after the 4-week application. Single-blinded-investigator (TN)

assessments were based on a 3-point scale (improved, no change, worse). The study protocol was approved by the Institutional Review Board of the Hokkaido University Hospital (approved No. 014-0427), registered in UMIN-CTR (http://www.umin.ac.jp/ctr/index.htm; UMIN000018677), and conducted after obtaining written informed consent by all participants or their legal guardians in compliance with the Declaration of Helsinki Principles.

Statistics

Statistically significant differences were determined by the Student's unpaired two-tailed *t-test* and the paired *t-test* for the comparison of mRNA expression levels and VAS scores, respectively. Data are represented as the mean ± the SEM.

CONFLICT OF INTEREST

The authors state that there are no conflicts of interest.

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Table 1. Summary of the NPPK patients included in this clinical study.

Patient	Age (y/o)	Gender	Mutation 1	Mutation 2	Left hand	Right hand	Previously reported?
P1	28	Male	c.796C>T	c.796C>T	Gentamicin	Vaseline	Yes*
P2	29	Male	c.796C>T	c.796C>T	Gentamicin	Vaseline	No
P3	6	Female	c.796C>T	c.455-1G>A	Vaseline	Gentamicin	Yes*
P4	40	Female	c.796C>T	c.455-1G>A	Vaseline	Gentamicin	Yes*
P5	46	Male	c.796C>T	c.796C>T	Gentamicin	Vaseline	Yes*

*Mizuno *et al*., 2014

FIGURE LEGENDS

Figure 1. Gentamicin suppresses the founder nonsense mutation c.796C>T in *SERPINB7 in vitro*. (a) Schematic diagrams of wild-type and mutant *SERPINB7* cDNA N-terminally tagged with *FLAG*, both driven by the CMV promoter. *Luc2*, encoding firefly luciferase (FLuc), was co-transfected as a transfection control. (b) Ribosomes usually stop protein translation at a PTC in the mutant *SERPINB7* mRNA, producing a 297-amino-acid (aa) truncated SERPINB7. However, readthrough drugs such as gentamicin promote synthesis of the 492-aa full-length SERPINB7. (c) Expression of full-length SERPINB7 was detected using the anti-FLAG antibody. Transient transfection of the mutant cDNA into AD293 cells resulted in little synthesis of full-length SERPINB7. However, gentamicin at a concentration of 312 μg/ml or higher promoted the cells to produce more full-length SERPINB7 in a dose-dependent manner. By contrast, ataluren did not increase the synthesis of full-length SERPINB7. G418 was used as a positive control. Note that gentamicin did not increase the protein levels of truncated SERPINB7 or FLuc. These experiments were performed more than three times. Representative data are shown.

Figure 2. Gentamicin-induced restoration of full-length SERPINB7 protein in NPPK keratinocytes. Western blotting analysis of NHEK cell lysates using an anti-N-terminal SERPINB7 antibody yielded a number of non-specific bands. siRNA knockdown of *SERPINB7* was used to identify SERPINB7-specific bands (red arrows). Treating NPPK patient-derived keratinocytes with 10,000 or 15,000 μg/ml gentamicin for 60 hours restored the production of full-length SERPINB7. G418 was used as a positive control. β-actin (ACTB) was used as a loading control. The band intensities of SERPINB7 normalized to those of ACTB were also shown. This experiment was performed more than three times. Representative data are shown.

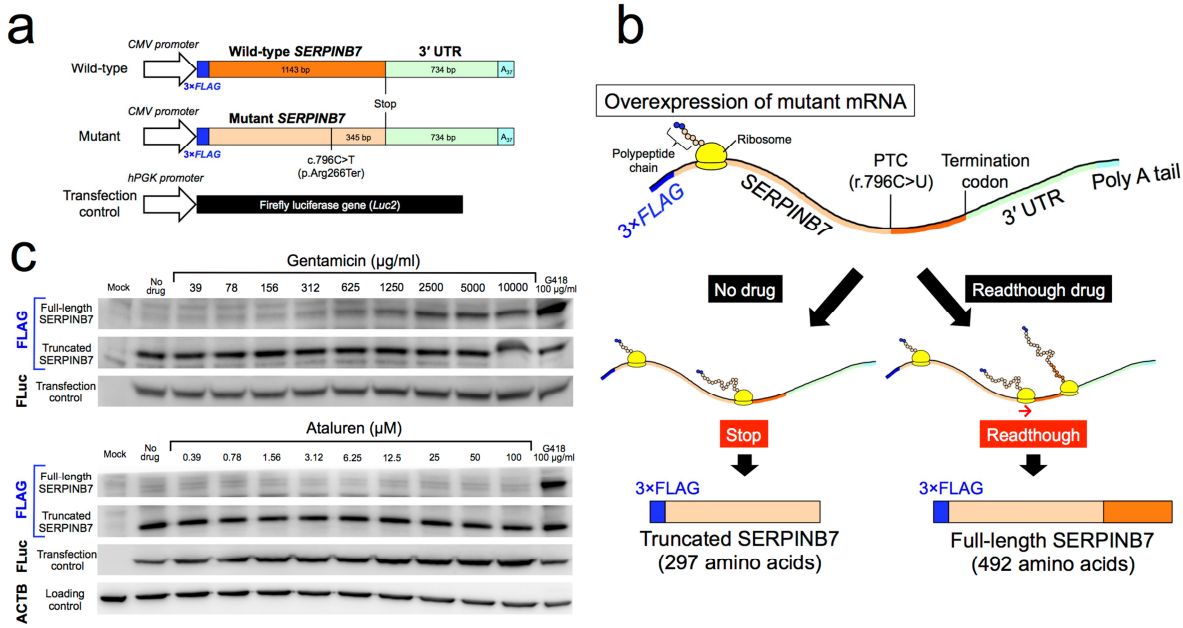
Figure 3. A "proof-of-concept" compassionate use study of topical gentamicin in NPPK patients. In this investigator-blinded, randomized, four-week compassionate use study, five NPPK patients with the nonsense mutation c.796C>T were randomly assigned to application of 0.1% gentamicin ointment on one hand and Vaseline on the other. (a) The patients reported improvement of hyperkeratosis on the gentamicin side

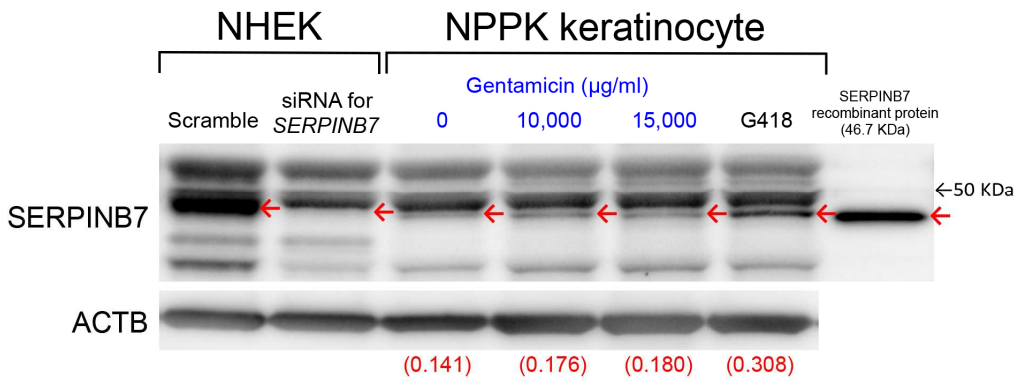
in visual analog scale (VAS) scores compared with the control side. The results were statistically significant (P=0.0349) by the paired *t-test*. (b) A blinded-investigator assessment revealed reduced hyperkeratosis on the gentamicin-applied hand (P1 in the Table 1), especially on the fingers (yellow square).

Figure 4. Co-translational NMD of mutant *SERPINB7* transcripts harboring r.796c>u. (a) Quantitative real-time reverse transcriptase PCR analysis of *SERPINB7* mRNA levels in NPPK patients. The expression levels of *SERPINB7* mRNA in the whole skin of NPPK patients who were homozygous for c.796C>T were significantly lower than those of the wild-type individuals (P=0.00069). (b) Cycloheximide treatment increased *SERPINB7* mRNA levels more efficiently in immortalized NPPK keratinocytes with a homozygous c.796C>T mutation than in immortalized NHEKs at a concentration of 2.0 μM or higher. (c) siRNA knockdown of *UPF1* in immortalized NPPK keratinocytes resulted in a significant increase in *SERPINB7* mRNA levels. Note that expression values were normalized to *18S rRNA*, and each value represents the mean ± the SEM for at least three independent experiments. The Student's unpaired

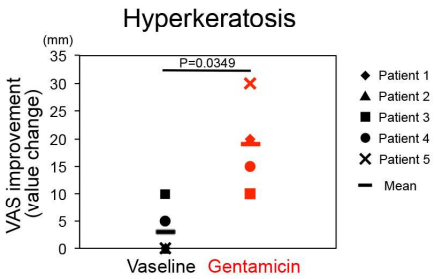
two-tailed *t-test* was used for statistical analysis.

Figure 5. 26S proteasome-mediated protein degradation of mutant SERPINB7 protein. (a) Transfection of the mutant and wild-type *SERPINB7* cDNA into HaCaT cells resulted in the production of truncated and full-length SERPINB7, respectively. Interestingly, the protein levels of truncated SERPINB7 were much lower than those of full-length SERPINB7. MG132 treatment increased the synthesis of the truncated protein in the mutant-transfected cells, while it did not alter the full-length SERPINB7 levels in the wild-type-transfected cells. Firefly luciferase (FLuc) and β-actin (ACTB) were used as a transfection and loading control, respectively. (b) Treatment of immortalized NPPK keratinocytes with MG132 promoted the production of truncated SERPINB7 in a dose-dependent manner. Note that cycloheximide (CHX) also increased truncated protein synthesis. ACTB was used as a loading control. Protein loading is also shown by Coomassie Brilliant Blue (CBB) staining.





a



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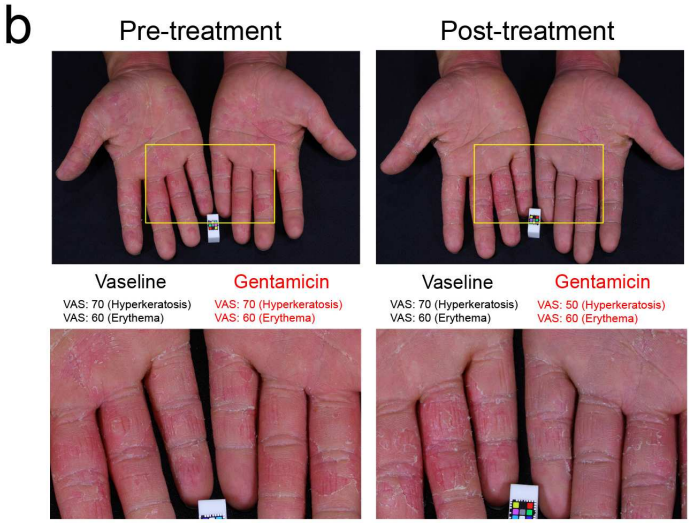

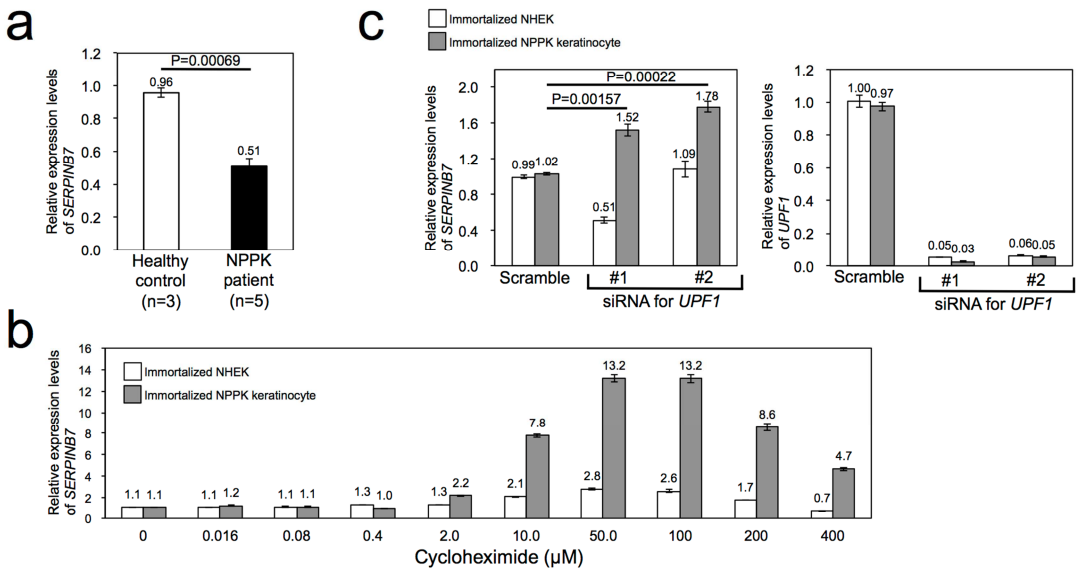
a
Relative expression levels of SERPINB7
P=0.00069
0.96
0.51
Healthy control (n=3)
NPPK patient (n=5)
c
Immortalized NHEK
Immortalized NPPK keratinocyte
P=0.00022
P=0.00157
0.99
1.02
0.51
1.52
1.09
1.78
Scramble
#1
#2
siRNA for UPF1
Relative expression levels of UPF1
1.00
0.97
0.05
0.03
0.06
0.05
b
Relative expression levels of SERPINB7
1.1 1.1
1.1 1.2
1.1 1.1
1.3 1.0
1.3 2.2
2.1 7.8
2.8 13.2
2.6 13.2
1.7 8.6
0.7 4.7
0
0.016
0.08
0.4
2.0
10.0
50.0
100
200
400
Cycloheximide (μM)